POTENTIAL OF *PLEUROTUS OSTREATUS* TO REMEDIATE DIESEL-CONTAMINATED SOIL IN SUBARCTIC MESOCOSMS

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A

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Abstract

Pleurotus ostreatus, a gilled basidiomycete, has previously been shown to biodegrade petroleum using extracellular enzymes. However, few studies have tested petroleum biodegradation by fungi, known as mycoremediation, in cold temperatures. I conducted mesocosm studies to assess the potential from mycoremediation of diesel-contaminated soil collected from interior Alaska with a cultivated strain of *P. ostreatus* var. *columbinus* at 4 ºC, 10 ºC, and 25 ºC. In soil, both uninoculated and inoculated with *P. columbinus,* diesel range organics (DRO) decreased by 22-28% ($p=0.455$), 41-55% ($p=0.236$), and 91-92% ($p=0.735$) at the three temperatures, respectively. The differences in DRO loss between uninoculated and inoculated mesocosms at each temperature were not statistically significant, most likely due to high soil heterogeneity. However, DRO loss was greater as temperature increased, and was significantly different between the temperatures evaluated. These results indicate that temperature is a more important factor controlling DRO loss than substrate or inoculation with *P. columbinus.* Inoculation may enhance DRO loss at medium temperatures, but inoculation does not appear to enhance DRO loss at the highest and lowest temperatures in this study. The results also suggest that manipulating the temperature of remediation sites may be more important than inoculating with *Pleurotus*, and that inoculation might not be needed at sites where temperature can be increased.

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General Introduction

Mycoremediation is the utilization of certain species of fungi to degrade or remove pollutants. Mycoremediation may be a cost-effective solution to contamination with a variety of different petroleum hydrocarbon mixtures, including crude oil, diesel, and other fuels in terrestrial environments. Oil spills are a pervasive issue in soils of northern climates, and cleanup is especially difficult in remote areas. The effects of cold temperature on mycoremediation are under-explored, yet they are important to the future success of mycoremediation in cold regions.

Chapter one summarizes the literature on mycoremediation of hydrocarbons in soil, and considers the potential for mycoremediation at colder temperatures. Mycoremediation shows potential for breaking down recalcitrant contaminants, but methods that would allow the most effective mycoremediation techniques are still being developed and tested. The chapter addresses the species, mechanisms, and ecological factors that play important roles in mycoremediation. In general, there is a lack of knowledge of the effects of cold temperature on mycoremediation. Finally, the second chapter of this thesis contains a study of diesel degradation by the gilled basidiomycete *Pleurotus columbinus* at a range of temperatures, including cold soils typical of Alaska field conditions.

Chapter 1 Mycoremediation of Petroleum: A Literature Review[1]

Introduction

Mycoremediation is one cleanup technique among the larger field of bioremediation. Bioremediation includes two different approaches, biostimulation and bioaugmentation. Biostimulation is the stimulation of pollutant-degrading microbes that are already present in the environment by aeration, nutrient addition, or changing the environmental conditions in other ways to optimize degradation. Bioaugmentation is the addition of microbes, fungi, or plants to the contaminated area to enhance degradation of target compounds. Bioaugmentation is used when the organisms required to degrade the contaminant are not present, or they are too sparse to be effective (EPA, 2014). Culturing and reinoculating with microbes that are already naturally available in the soil is thought to be more effective than adding commercially available cultivars (Hosokawa *et al.,* 2009), because native organisms are presumed to be well suited to other components of their environment. In the context of mycoremediation, fungi isolated from the environment can be tested to determine whether they may be used for bioaugmentation, following studies of indigenous contaminant-degrading microbes could reveal whether bioaugmentation is required.

History

The ability of fungi to break down phenols and aromatic hydrocarbons in wood has been known for decades. In the 1960s, Dr. Horst Lyr described ligninolytic degradation by white rot

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[1] Anderson, C. Mycoremediation of Petroleum: A Literature Review. Prepared for submission in the *Journal of Environmental Science and Engineering*.

fungi (Lyr, 1963). White rot fungi are wood decomposers that preferentially degrade lignin, leaving behind pulpy, white cellulose and hemicellulose. Bumpus (1985) expanded on these initial findings, hypothesizing that white rot fungi could degrade persistent xenobiotics since they have carbon skeletons similar to lignin. Bumpus evaluated the chemical breakdown of polychlorinated biphenyls, trinitrotoluene (TNT), dioxins, and lindane by *Phanerochaete chrysosporium*.

Phanerochaete chrysosporium was the first popular white rot fungal species used to degrade pollutants. Most ensuing mycoremediation research initially evaluated *Phanerochaete chrysosporium* and its closely related species. White rot fungi are believed to be the only group of organisms capable of completely mineralizing lignin (Anastasi *et al.,* 2013). However, further research has revealed that many different groups of fungi are capable of at least partially degrading lignin and similar hydrocarbons, including the coprophilic *Agaricus bisporus* (ten Have *et al.,* 2003), the mycorrhizal *Glomus caledonium* (Chen *et al.,* 2009), the airborne *Aspergillus fumigatus* (Bento *et al.,* 2005), the black yeast *Exophiala xenobiotica* (Isola *et al.,* 2013), a lichen (Lee *et al.,* 2005) and many other species (Anastasi *et al.,* 2013; Davies and Westlake, 1979; Harms *et al.,* 2011; Lee *et al.,* 2005; and Pinedo-Rivilla *et al.,* 2009).

As investigations of mycoremediation have proceeded, the breadth of pollutants fungi are known to degrade has also increased. In addition to petroleum and polycyclic aromatic hydrocarbons, white rot fungi were also found to degrade explosives, pesticides, polychlorinated biphenyls, synthetic dyes, and creosote (Pointing, 2001). Fungi that could live on the pollutant alone, without co-metabolism, were discovered subsisting on volatile organic hydrocarbons (Isola *et al.,* 2013) and polyurethane (Russell *et al.,* 2011).

As documentation of additional fungal species and pollutants they degraded grew, a new field emerged, and the term "mycoremediation" was coined in 2005 by Paul Stamets. Mycoremediation refers to any method of detoxifying pollutants using fungi. The concept of mycoremediation came to popular public attention as a result of several TED talks by Stamets and the creation of the website and book "Radical Mycology" (McCoy, 2016).

Species evaluated for mycoremediation potential

Many oil-degrading fungi have been isolated from the environment, some of which are even more effective than bacteria in completely degrading crude oil (Cerniglia and Perry, 1973; Davies and Westlake, 1979). More recent studies have found that microfungi such as ascomycetes (Bento *et al.,* 2005), *Cladosporium sp.* (Giraud *et al.,* 2001), *Penicillium sp.* and other deuteromycetes (Prenafeta-Boldu *et al.,* 2001) isolated from the environment can degrade hydrocarbons. However, in nearly all of these studies of fungal degradation of petroleum products, the species examined were microfungi. Most white rot fungi are basidiomycetes, which consist of a mycelial network that can draw water and nutrients throughout the substrate. In addition, the structure of basidiomycete mycelia allows this fungal group to bridge gaps between patches of pollutant, instead of requiring continual contact with the pollutant as a growth substrate (Harms *et al.,* 2011). For pollutants that are patchily distributed, the ability to co-metabolize the pollutant and another substrate is an advantage.

Pleurotus ostreatus has many desirable properties as a species for mycoremediation. It is a versatile white rot basidiomycete that has proven successful in degrading petroleum in many studies (Leonardi *et al.,* 2006; Márquez-Rocha *et al.,* 2000; Meysami and Baheri, 2003; Novotný *et al.,* 2004; Novotný *et al.,* 1999; Okparanma *et al.,* 2011; Young *et al.,* 2015; Zitte *et al.,* 2012).

Pleurotus ostreatus produces a wide range of enzymes active against a broad range of compounds. *P. ostreatus* has the ability to transport water, nutrients, bacteria and the contaminants themselves along the mycelium, sharing resources for contaminant degradation throughout the soil matrix. Finally, *P. ostreatus* has the ability to span air-filled gaps in soil and penetrate micropores, and co-metabolize non-toxic organic molecules, allowing the fungus to remain active in conditions hostile to bacterial growth (Wick *et al.,* 2010).

However, the application of *P. ostreatus* in mycoremediation has a number of disadvantages. First, the fungus requires an aerobic environment. Second, once established, the mycelia are sensitive to disturbance. Standard methods of bioremediation that aerate by tilling are designed for bacteria, and would damage the mycelium by breaking it up. Lastly, *Pleurotus ostreatus* requires a lignocellulose amendment, since it is a wood rotting fungus, and may not compete well with native fungi and bacteria in the soil without its principle substrate (Harms *et al.,* 2011).

A mycoremediation study involving *P. ostreatus,* popularized by Paul Stamets in *Mycelium Running* (2005) involved layering diesel-contaminated soil with *P. ostreatus* straw spawn in large, 10 cubic yard mounds covered in shade cloth. Other mounds were mixed with nitrogen fertilizer and left uncovered, or inoculated with bacteria plus nitrogen fertilizer and covered with polyethylene tarps. Over 16 weeks, the mycelienated mounds lost their oily smell, turned lighter in appearance, and began to grow vascular plants and secondary decomposer fungi. The control and other treatments did not significantly change appearance or smell. Although this informal study showed potential, important questions remained. There was such heterogeneous diesel distribution in the soil that the authors could not conclude that there was any difference between the treatments when testing for total petroleum hydrocarbons. Analysis

of individual PAHs was initiated, but not completed due to a lack of funding (Thomas *et al.*, 1998).

A more carefully controlled laboratory study by Novotný *et al.* (1999) found that *P. ostreatus* degraded PAHs better than two other white rot fungi tested. Anthracene, pyrene, and phenanthrene decreased by 81-87 percent, 84-93 percent, and 41-63 percent in 2 months, respectively, with the *P. ostreatus* treatment. Subsequent lab and bench studies chose *P. ostreatus* as an organism for bioremediation of PAHs (Márquez-Rocha *et al.*, 2000; Leonardi *et al.*, 2006). Another study of mycoremediation with *P. ostreatus* reported an 85 percent to 90 percent loss of total petroleum hydrocarbons after 4 weeks of incubation, but lacked any statistical analysis verifying that petroleum loss was within a 95% confidence interval (Zitte *et al.*, 2012).

Another set of mycoremediation studies capitalized upon the need to dispose of fungal spawn as a waste byproduct of mushroom cultivation. Using two applications of waste *P. ostreatus* spawn to contaminated soil, Eggen and Šašek (2002) saw an 87 to 99 percent degradation of PAHs in 12 weeks, and Okparanma *et al.* (2011) observed an 80 to 92 percent PAH loss over 8 weeks. Gasecka *et al.* (2013) used spent *Agaricus bisporus* and *Lentinula edodes* compost to degrade PAHs by 63 percent to 87 percent in 12 weeks. Mycoremediation appears to offer an excellent use of aged fungal spawn that would otherwise be wasted by the mushroom cultivation industry.

In general, those mycoremediation studies examining the degradation of specific PAHs have found significant modification and mineralization by fungal treatments (Gasecka *et al.*, 2013; Márquez-Rocha *et al.*, 2000; Novotný *et al.*, 1999). However, three mycoremediation studies measuring total petroleum hydrocarbon degradation (Meysami and Baheri, 2003, Thomas *et al.*, 1998; Zitte *et al.*, 2012) had too much uncertainty to determine whether there was a

significant difference between the fungal treatments and controls.

Enzymes

In examining the potential for mycoremediation, a key issue is the mechanism by which the fungi achieve the breakdown of pollutants. The common ligninolytic enzymes in white rot fungi are laccases and peroxidases (Elisashavili *et al.,* 2008). They are both extracellular proteins that catalyze hydrocarbon degradation using free radicals, molecules with single unpaired valence electrons. Laccases oxidize oxygen to release a free radical, and peroxidases oxidize hydrogen peroxide, Mn^{2+} , or Mn^{3+} to release free radicals (Harms *et al.,* 2011). The free radicals indiscriminately cleave bonds, breaking straight chain hydrocarbons or opening aromatic rings, thereby making hydrocarbons more bioavailable for uptake and biodegradation by fungi, bacteria and other degraders (Meulenberg *et al.,* 1997). In fungi, some metabolites are also taken into the hyphae and further degraded with cytochrome P450 (Harms *et al.,* 2011). With their cocktail of non-specific enzymes, white rot fungi are some of the only organisms able to completely mineralize hydrocarbons (Anastasi *et al.,* 2013).

Performance of laccases and peroxidases can be affected by environmental conditions. Lignin degradation appears to be stimulated more as a response to nutrient depletion than to the presence of pollutant (Madhavi and Lele, 2009). Ligninolytic enzyme production is greatest during the fungal vegetative growth stage and decreases sharply during fruiting (Elisashavili *et al.,* 2008). Laccase activity can be increased by warm temperatures (Madhavi and Lele, 2009). Laccase production can be inhibited by low availability of copper, since copper is an important constituent of laccase (Tychanowitcz *et al.,* 2006). However, not all laccases contain four copper atoms; one isolated from *P. ostreatus* contained one copper atom, one zinc atom, and two iron atoms instead (Palmieri *et al.,* 1997).

Incomplete mineralization can yield toxic metabolites (Atlas, 1995; Pinedo-Rivilla *et al.*, 2009). Quinones are common metabolites of PAHs, and some can be more toxic than the initial compound. Ligninolytic enzymes can further mineralize quinones, or make them more bioavailable to degradation by bacteria (Pozdnyakova, 2012). The intracellular cytochrome P450 enzymes are also able to degrade smaller metabolites into completely mineralized carbon dioxide, water, and proteins (Harms *et al.*, 2011).

Synergy with bacteria

Although white rot fungi are able to completely mineralize hydrocarbons, more commonly they perform only the initial attack, breaking open hydrocarbon chains and rings to make the compounds more available to microbial decomposition. During a 60 day study of petroleum bioremediation, Chen *et al.* (2009) found that *Glomus caledonium* and *Bacillus subtilis,* used together, removed 92.6 percent of total petroleum hydrocarbon while indigenous microorganisms removed 21.9 percent, and the combined effect was greater than the sum of individual removal by each in pure culture. In some species, the mycelia form rhizomorphs that can conduct water throughout the soil, carrying bacteria to otherwise inaccessible areas (Kohlmeier *et al.* 2005). Li et al. (2008) observed a synergistic effect of *Mycobacterium* and filamentous fungi, with diesel loss increasing from 20.9 percent and 34 percent separately to 99 percent together over a 5-day treatment.

Temperature

Most studies on mycoremediation were conducted at room temperature (~25°C). No studies to my knowledge have specifically evaluated the effect of a range of temperatures,

including low temperatures characteristic of ambient condition in northern soils, on mycoremediation. However, studies on other methods of bioremediation at different temperatures can be useful for guiding mycoremediation design.

Low temperatures inhibit bioremediation of hydrocarbons due to their lower volatility and reduced microbial metabolism (Atlas, 1981; Horel, 2009; Margesin and Schinner, 2001; Yang *et al.,* 2009). The average optimal temperature for standard bioremediation is considered to be 30°C (Das and Chandran, 2011), although bioremediation at cold temperatures is possible. A study on sub-Antarctic soils found that even at 4°C, a treatment with fertilizer was associated with a 75 percent reduction of diesel in 180 days. Additionally, at the conclusion of the study, soil toxicity was lower at 4°C than at 10°C or 20°C (Coulon *et al.,* 2005). In Antarctic soils at 4°C, biostimulation with nutrients was effective in degrading 98 percent of alkanes within 18 weeks. In that study, augmentation with native bacteria was only effective at removing 45 percent of alkanes, most likely due to nutrient limitations. In cold Antarctic soils the more toxic PAHs remained longer than 12 weeks (Stallwood *et al.,* 2005). Naphthalene, the lightest PAH, was mineralized at a rate of 4.02 percent per day at 8°C with nitrogen fertilization in Antarctic soil (Aislabie *et al.,* 1998). In general, relatively little is known about PAH biodegradation at cold temperatures in soil (Margesin and Schinner, 2001).

A few mycoremediation field studies have been conducted over a long enough period of time to include cold temperature seasonal periods. Ambient temperature at a study using *Phanerochaete sordida* on creosote sludge fluctuated between 21°C and 38°C for the first 45 days, then dropped to 15°C and below. Even with cooler temperatures, PAH depletion was more rapid in fungal-treated soil than in controls (Davis *et al.,* 1993). Another study in Finland using *P. velutina* had temperatures ranging from 6°C to 23°C inside the soil piles (Winquist *et al.,* 2014). In the lab scale, fungal amendment enhanced degradation of PAHs, but not in the field scale. The

authors hypothesized that since there was a lower PAH concentration in the field scale than in the lab (1400 mg/kg versus 3500 mg/kg), the field soil was found to be less toxic and native bacteria were better able to perform bioremediation. There was a dramatic improvement in quality in field soils over six weeks. In spite of the cool temperatures, the entire bioavailable fraction of PAHs was degraded in three months.

In summary, the available literature indicates that white rot fungal amendment to soil along with a lignocellulose source may enhance petroleum degradation at low temperatures. The addition of limited nutrients, especially nitrogen, also appears to speed up bioremediation at low temperatures.

Mycoremediation in the context of bioremediation methods

The method used for treating petroleum spills is determined by soil type. Bioremediation methods can be either in-situ or ex-situ. Since they require excavation, ex-situ methods are usually an order of magnitude greater in cost than in-situ methods.

A widely used bioremediation method is land farming, which involves excavating the soil and spreading it out over a sealed base, then aerating and fertilizing it to promote biodegradation. Biopiling is another standard ex-situ method in which soil is heaped and mixed with some kind of bulking agent like crop residue or bark, and sometimes nutrients or microorganisms (Williams, 2006). Biosparging or venting is an in-situ method of injecting air or nutrients into the soil at the contaminated site to stimulate microbes (Khan *et al.,* 2004). Phytoremediation is an experimental method of planting vegetation in contaminated soil to stimulate microbial degradation in the rhizosphere and facilitate extraction of soluble or volatile contaminants (Khan *et al.,* 2004).

Williams (2006) observed that the use of bioremediation is growing rapidly, as it is relatively low-cost and involves less drastic modification of the environment than standard

treatments. Land farming ranges from $50-$75/ton of soil, while biopiling is $8-$25/ton, biosparging costs $10-$75/ton, and phytoremediation ranges from $10-$480/ton (Williams, 2006; Khan *et al.,* 2004). Mycoremediation in Washington state is estimated to cost $13.5/ton, excluding the cost of monitoring and lab studies (Thomas *et al.,* 1998).

Twin studies by Cajthaml *et al.* (2002) and Bhatt *et al.* (2002) concluded that composting is "substantially more efficient in removing all PAHs, including higher molecular weight ones, than fungal treatment". However, the two studies differed in some important respects, raising the question of whether they are indeed comparable. The Cajthaml study used a higher ratio of amendment to soil than the Bhatt study. Cajthaml mixed 2832 mg/kg PAH-contaminated soil with compost consisting of wheat straw, chicken manure, and gypsum, at a ratio of 1:4 soil to compost. Bhatt used a ratio of 2.5:1 soil to spawn. The Cajthaml treatment was likely better aerated. The compost chamber dimensions were 1.3 x 1.35 x 2.5 m, with aeration tubes. Bhatt et al. (2002) layered the same contaminated soil with straw colonized with mycelium in one liter Erlenmeyer flasks. The Cajthaml treatment was large enough to achieve a thermogenic effect, as opposed to Bhatt's smaller treatment. Temperatures in Cajthaml et al.'s composting study exceeded 60ºC in the first 6 days, and then gradually cooled as metabolism slowed over the next 36 days versus a steady 26ºC for 14 weeks in Bhatt's study. In the Cajthaml study, composting yielded a 42 to 68 percent degradation of 3-4 ring PAHs and 35 to 57 percent degradation of higher mass PAHs, whereas in the Bhatt study, flask mycoremediation yielded 13 to 62 percent degradation of 3-4 ring PAHs and no reported analysis of higher molecular weight PAHs. An experiment comparing larger-scale compost treatments with and without addition of fungal spawn would control for temperature differences and better address the question of whether mycoremediation enhances petroleum degradation.

Some studies indicate that composting does enhance petroleum degradation compared to

conventional land-farming techniques. Guerin (2000) found that composting resulted in 50 percent loss of PAHs, whereas land farming with or without added organic matter only resulted in 5 percent loss. The greater rate of PAH loss in the compost treatment was associated with higher temperatures. The compost treatment reached higher temperatures, up to 42ºC, than the land farming treatment, and remained stable day and night. The maximum temperatures in the land farming treatments were 26ºC and 27ºC. In the lab-scale compost treatments, soil temperature did not exceed 28ºC (room temperature) because the thermal mass was too small to allow thermogenesis. Organic matter was reduced over time, and the proportion of lignin detected by the Klasson method (acid soluble lignin) increased, indicating a paucity of lignin-degrading fungi.

Conclusion

Warming the ground is one of the most effective treatments for promoting biodegradation, especially in cold climates, and heaping or composting can be a cost-effective way to warm the substrate (Filler and Carlson 2000, Semple *et al.* 2001, Williams 2006). In such a favorable, warm environment, amending with ligninolytic fungi could potentially enhance degradation of toxic pollutants.

The thermogenesis that occurs during some forms of mycoremediation may be an important factor in accelerating microbial biodegradation overall, indicating that temperature is the most important factor in enhancing diesel loss. Research on the success of mycoremediation at variable temperatures, especially cold temperatures, must still be done. The rate of petroleum degradation in cold regions is problematically slow, and a method to enhance and accelerate petroleum degradation is highly desirable.

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Chapter 2 Bioaugmentation of Diesel-Contaminated Soil with the White-rot Fungus, *Pleurotus ostreatus,* in Sub-Arctic Mesocosms[1]

Introduction

When Alaska North Slope oil was discovered in the late 1960's, a boom of infrastructure occurred throughout the state. Petroleum fueled the construction of airstrips, schools and clinics. Accompanying tank farms were built to store fuel for transportation and heating during long intervals between barge deliveries. Spills during the delivery and consumption of oil, such as corroding storage tanks, overtopping, seal failures and human error, constitute a significant health hazard to communities and a problem for the state. An average of 86,300 gallons of diesel alone were spilled in Alaska every year in the past 7 years, equivalent to contaminating over 323,000 tons of soil per year (ADEC, 2014). Cleanup costs vary considerably by the size, type and location of spill, ranging from $15,000- $25,000 for cleaning up a residential spill (Starsman, personal communication) to $2.5 billion for the Exxon Valdez spill, not counting billions more from fines, penalties, and lawsuits (Lyon and Weiss, 2010). This history has created the demand for inexpensive, rapid remediation of petroleum spills throughout Alaska.

Current remediation methods can be environmentally and economically costly. In Alaska and Canada, petroleum-contaminated soil is often trucked to a facility for thermal desorption to vaporize or combust contaminants, or "encapsulated" by paving over (Filler and Carlson, 2000, Guritz, 2012). Transporting contaminated soil from rural Alaska to such facilities is prohibitively expensive. In addition, encapsulation removes the utility of the soil. Bioremediation, restoring the soil by treating pollutants with natural methods, offers the promise to overcome these problems, and is preferred by regulatory agencies (EPA, 2014).

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[1] Anderson, C. Bioaugmentation of Diesel-Contaminated Soil with the White-rot Fungus, *Pleurotus ostreatus,* in Sub-Arctic Mesocosms. Prepared for submission in *Fungal Ecology.*

Properties and hazards of diesel

Before a bioremediation method is chosen, properties of the contaminant being degraded must be examined. Diesel petroleum is a complex mix of hydrocarbons, composed of straight chains and aromatic rings of carbon atoms, often with sulfur or other elements (ATSDR, 1995). Diesel is classified as an irritant to skin and eyes, and may cause mild kidney and lung damage. It is easily absorbed into the body and some components of diesel, like polycyclic aromatic hydrocarbons (PAHs) are carcinogenic (DOL, 2006). PAHs make up only less than 5% of diesel fuel (DOL, 2006), but are the most toxic (Irwin, 1997), and some of the most difficult components to biodegrade (Atlas, 1995).

A new field known as "mycoremediation" is emerging, a term coined by the mycologist-businessman Paul Stamets. The concept is that certain fungi are able to break down a wide variety of contaminants using the same enzymes used to metabolize lignin. Laccases, peroxidases, and cytochrome P450 monooxygenase are commonly utilized by ligninolytic fungi.

German phytopathologist Lyr (1963) was the first to observe that fungi can break down aromatic compounds. Bumpus expanded on that knowledge and suggested that lignin-degrading enzymes may be used for site decontamination, showing degradation of DDT, PCBs, dioxins, and lindane using *Phanerochaete chrysosporium* in the lab (Bumpus, 1985). In recent years, scientists have evaluated fungal degradation of polycyclic aromatic hydrocarbons (PAHs) (Bhatt *et al.,* 2002; Gasecka *et al.,* 2013; Giraud *et al.,* 2001; Leonardi *et al.,* 2006; Meulenberg *et al.,* 1997), DDT (Purnomo *et al.,* 2011), TNT and other explosives (Fritsche, 1998, Gottschalk, 1998), crude oil (Davies and Westlake, 1979; Hadibarata and Tachibana, 2009; Isikhuemhen *et al.,* 2003) and many other hazardous pollutants (Harms *et al.,* 2011). In comparing three ligninolytic species, *Pleurotus ostreatus* produced the highest quantity of laccase enzymes and

most effectively degraded PAHs (Novotný *et al.,* 2004). Although progress has been made to validate mycoremediation in the laboratory, there is a need for field-scale testing before this technology can be compared to other decontamination methods (Novotný *et al.*, 2004; Pinedo-Rivilla *et al.,* 2009; Winquist *et al.*, 2014). Rural Alaska villages appear to be an ideal location for such field testing, since traditional forms of remediation are impractical and they have aging petroleum-dependent infrastructure.

In the Koyukon Athabascan village of Kaltag, diesel contamination at the site of a tank farm serving the school was reported to the Alaska Department of Environmental Conservation (ADEC) in 1991 (Guritz, 2012). All tanks were removed from the area in 2002. Most of the contaminated soil was excavated and spread out to a depth of 18 inches during the summer of 2014 in preparation for land-farming and experimental phytoremediation by the Leigh lab at the University of Alaska, Fairbanks (Shannon & Wilson, 2014). Soil from the Kaltag School Oil Seep was used in this study to assess the ability of *Pleurotus ostreatus* var. *columbinus* to degrade diesel in mesocosms designed to simulate the subarctic environment, particularly cold soils.

Established mechanisms of mycoremediation

Multiple fungal taxa have shown the ability to degrade harmful compounds (Singh, 2006), but most research has been focused on ligninolytic fungi, also called white-rot fungi for the color of the de-lignified cellulose and hemi-cellulose substrates they leave behind. The main enzymes white-rot fungi use to degrade lignin, laccases and peroxidases, are extracellular (Anastasi *et al.,* 2013; Novotný *et al.,* 2004). Laccases and peroxidases are non-specific, using free radical reactions to cleave bonds (Bumpus, 1985; Christian *et al.,* 2005). Laccases contain copper, and use oxygen to induce one-electron oxidation of organic compounds. Peroxidases use

H2O2 to induce one-electron oxidation of aromatic compounds, or some oxidize Mn^{2+} to Mn^{3+}, which then oxidizes organic compounds (Harms *et al.,* 2011). Extracellular enzymes may be dispersed by water throughout contaminated sites. Rhizomorphs, root-like bundles of fungal hyphae, can transport water with oil-degrading bacteria as far as the mycelium extends (Kohlmeier *et al.,*

2005). The main mechanism of hydrocarbon degradation by bacteria is intracellular, but bacteria can also produce enzymes, or immobilize and disperse petroleum by physically adhering to the pollutant's surface or by producing surfactants (Das and Chandran, 2011). Data from several studies suggest that fungi and bacteria can degrade contaminants synergistically (Chen *et al.,* 2009; Li *et al.,* 2008; Tornberg *et al.,* 2003; Atlas, 1995), with fungi breaking down larger molecules, making them bioavailable to bacteria (Meulenberg *et al.,* 1997). The addition of *Botrysphaeria rhodina* and *Pleurotus pulmonarius* to soil were shown to increase bacterial diversity, an indicator of soil health (Federici *et al.,* 2007).

Effects of temperature on petroleum degradation

Cold temperatures decrease volatility and increase sorption of petroleum compounds, as well as slowing biological metabolism (Yang *et al.,* 2009). Natural attenuation is slow in northern climates (Atlas, 1995; Yang *et al.,* 2009), so some form of stimulation is desirable to speed the process. Biostimulation using added nutrients to clean petroleum contamination at cold temperatures has been demonstrated successfully in previous studies (Stallwood *et al.,* 2005), with best soil fungi performance at 10°C (Mair *et al.,* 2013). Few studies have explored the effect of cold temperatures on mycoremediation.

Rationale for use of Pleurotus *in mycoremediation*

Pleurotus columbinus was chosen because it is a white-rot fungus, and the species is cold-tolerant (Fungi Perfecti, personal communication), so it is more likely to succeed in Alaska's cold climate. Initially, I also sought to evaluate and compare mycoremediation potential of a native white-rot fungal species, but I was unsuccessful in isolating native *Pleurotus* cultures. *Pleurotus columbinus* was obtained from Fungi Perfecti (Olympia, WA) for experimental use. It arrived growing on mycelienated dowels and was expanded to straw and sawdust. Mycelienated substrate is called "spawn" by mushroom cultivators. Fungi Perfecti identifies the origin of the cultures they sell as the "woodlands of the Pacific Northwest". *Pleurotus columbinus'* ability to tolerate petroleum-contaminated soils was tested before the degradation experiment (Matsubara *et al.,* 2006).

A body of evidence shows that pure culture fungal or bacterial inoculum has a low survival rate when used in contaminated natural soils (Atlas, 1995; Laine and Jørgensen, 1996; Lladó *et al.,* 2013). To help white-rot fungi establish in the soil, it is recommended that wood chips, straw, or some other bulky, lignocelluose substrate is added (Meysami and Baheri, 2003). Layering contaminated soil with mycelienated substrate has yielded success (Bhatt *et al.,* 2002). In this study, I use layers of *P. columbinus* straw and sawdust spawn to inoculate the contaminated soil.

Methods

Examination of diesel tolerance of Pleurotus columbinus

To determine sensitivity of *P. columbinus* to diesel, I used a solid media method (adapted from Hadibarata and Tachibana, 2009), in which I measured the diameter of mycelial growth on

malt extract agar plates containing different concentrations of diesel. I vacuum filtered diesel with a 90 mm filter, then pipetted onto the surface of BD malt extract agar plates in 1 mL, 0.5 mL, 0.1 mL, 0.05 mL, 0.01 mL and 0 mL aliquots, and spread with a glass spreading tool. I tested four replicates of each concentration of diesel and three replicates of negative control. One square cm. of mycelienated agar was placed in each plate and incubated at 25ºC for 2 weeks. Mycelial growth diameters were measured at 0, 1, 3, 4, 8, & 9 days to determine petroleum tolerance. The experiment was repeated with the diesel mixed into warm malt extract agar instead of spreading it on top, and mycelium diameter was measured at 5, 7, 10, & 13 days.

Propagation of mycelium

To expand mycelium to a quantity required for remediating contaminated soil, I began by placing the mycelienated dowels on agar plates of Sabourad agar, potato dextrose agar, and malt extract agar. After the plates were covered with fungal growth, I used ten 1-cm plugs of mycelienated agar to inoculated three liters of whole oat grains. The oats had been soaked overnight in water, drained, and then autoclave-sterilized at 121ºC for 45 minutes and cooled before inoculation. The mixture was incubated in glass quart-jars until colonization and growth was complete (all oats visible through the glass covered in mycelium), at about 4 weeks. Next, 12 L of soaked straw was sterilized in autoclave bags at 212ºC for one and a half hours. The straw was inoculated with the prepared oat spawn and then incubated between 10ºC and 20ºC until colonization was complete (about 4 weeks). This sequential process of growing mycelium on plugs, oats, and then straw speeds expansion of the mycelium, since it is easier for mycelium to colonize more closely packed substrate (Royse and Sanchez-Vazquez, 2001; Stamets, 1993).

Collection and treatment of diesel-contaminated soil

Diesel-contaminated soil was collected from the Kaltag School Oil Seep during the first two weeks of August 2014. Soil was stored in sealed plastic buckets and stored at 4°C until experiment initiation. Soil was homogenized with a 5 mm sieve during the three days preceding the start of incubation. Before incubation, moisture content was determined gravimetrically. The contaminated soil was a silty loam (sand 27%, silt 56%, clay 17%). Carbon, nitrogen, potassium, phosphorus, and pH were measured by the UAF Palmer Research Center (Table 1).

Table 1. Description of soil characteristics

Site	pH	C	NH^{+}_{4}-N	NO^{-}_{3}-N	P	K	Cu	Mn	CEC	Sand	Silt	Clay
		%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	meq/100g	%	%	%
Kaltag 10-28-14	5.37	22.46	4	<1	21.9	28.2	1.3	104.0	15.59	27.0	56.0	17.0

[a]Initial moisture content: 24.8% based on 100 C oven dry wt.

Clean soil was also collected about 1/2 mile from the spill site, but not used in this study due to time constraints. Jessica Starsman's research on the same site confirmed that there were no diesel range organics (DRO) in uncontaminated soil (Personal communication).

Preparation of soil mesocosms

Thirty-nine one-liter glass canning jars were sterilized and layered with approximately 80% contaminated soil and 20% amendment by volume. The amendments consisted of either straw spawn, sawdust spawn, uninoculated sterilized straw or uninoculated sterilized sawdust (autoclaved for 45 minutes). Birch sawdust was obtained from the Alaska Bowl Co. (Fairbanks,

AK) and straw was purchased from the Alaska Feed Company (Fairbanks, AK). A ratio of 80% soil to 20% mycelienated substrate was used, recommended by Gasecka *et al.* 2013 for mycoremediation of PAHs.

Three replicates of each treatment (contaminated soil mixed with straw spawn, sawdust spawn, uninoculated sterilized straw, or uninoculated sterilized sawdust, Table 2) were incubated at 4°C, 10°C and 25°C. As an additional control to monitor natural attenuation, a one-liter jar of unamended soil was incubated at each temperature, 4°C, 10°C and 25°C. Moisture was monitored with a FieldScout TDR 100 soil moisture meter. Water (10-20 mL) was added when the volumetric water content dipped below 5%.

Table 2. Description of treatments

Treatment	Soil mesocosm composition
1	Diesel-contaminated soil + sterilized straw (3)
2	Diesel-contaminated soil + sterilized sawdust (3)
3	Diesel-contaminated soil + *Pleurotus* mycelienated straw (3)
4	Diesel-contaminated soil + *Pleurotus* mycelienated sawdust(3)
5	Diesel-contaminated soil without amendments (1)

[a]Treatments 1-5 were repeated in 25°C, 10°C, and 4°C incubation.

Quantifying mycelial growth

I created a qualitative growth rating system (Table 3) and monitored mycelial growth that was visible through the glass jars throughout the experiment.

Table 3. Mycelial growth ranking system

Mycelial growth ranking	Mycelial characteristics
0	No growth
1	Mycelium is barely alive or contamination is just starting
2	Mycelial growth all over substrate, but not fanning into soil
3	Mycelium is fanning into soil
4	Mycelium visible in all areas of the soil/glass interface
5	Mycelium dominates the jar, soil is less than 40% visible

[a]Treatments 1-5 were repeated in 20°C, 10°C, and 4°C incubation.

Quantification of Diesel Range Organics

Ten-gram composite soil samples were taken from each of the jars at 0, 1, 2, 4, 8, 12 and 16 weeks; sampling from the same jars each week. Using a scoopula, soil from each layer was removed in three to five scoops until the sample weighed approximately ten grams. Pieces of sawdust or straw that were accidentally scooped were excluded from the sample. To arrest diesel loss, samples were frozen in tightly capped 40 mL VOA vials (Thermo Fisher) at -80°C until the end of the experiment. Extraction was performed according to the Leigh Lab DRO standard operating procedure (Appendix). Next, 10 g. of contaminated soil were weighed into a 4 oz. amber glass jar. About 10 g. of sodium sulfate from BDH was added to dry the soil and stirred until the consistency of sand. Then, 0.5 mL of naphthalene-D8 (Sigma-Aldrich) was pipetted onto the soil sample and incorporated by stirring. Naphthalene-D8 is the surrogate, which is added to every soil sample to gauge extraction efficiency. Next, 50 mL of methylene chloride from BDH was added, and the jars were placed on a shaker table for three hours. After samples

settled, extracts were pipetted with glass transfer pipettes from VWR into a 40-mL VOA vial and frozen at -20° pending further analysis (Appendix). Extractions were completed between Dec. 3, 2014 and Feb. 3, 2015, 1 day to 71 days after sampling.

To prepare samples for gas chromatography (GC) and mass spectroscopy (MS), 1 mL of each sample was pipetted into a separate GC vial. Twenty-five µL of ~1,000 mg/L nitrobenzene-d5 (Sigma Aldrich) was added to each sample as an internal standard. Four 1 mL samples with known concentrations of diesel range organics and a 1 mL blank of methylene chloride were also analyzed (Appendix, Section 2). These standards were spiked with the same concentration of naphthalene-D8 as samples should have with 100% extraction efficiency. Analyses with GC and MS were conducted between Jan. 16, 2015 and Feb. 6, 2015. The column used was a 30.0 m x 320 µm x 0.25 µm Restek column with 5% diphenyl and 95% dimethyl polysiloxane stationary phase. GC/MS was performed between 45 and 218 days after extraction.

Injection settings

A splitless injection using helium as a carrier gas was used, with a 1.0 µL injection volume, inlet temp of 280°C, 4.80 psi pressure, and a flow of 44.3 mL/minute. A column flow of 1.7 mL/min. was maintained throughout the analysis. Initial temperature was 30°C for 1 min., then increased by 10°C per min. to 260°C (24 minutes). Temperature increased by 100°C per min. to 320°C and held for 5 min. (29.6 minutes). Total run time was 54.6 minutes. A solvent delay of 5.5 minutes was used to prolong the life of the detector. The mass spectrometer was set to monitor all ions between m/z 50 to m/z 550.

The total DRO area under the curve and the areas of three ions from nitrobenzene-D5 and one ion from D8 naphthalene were obtained from the chromatogram. The DRO concentration

was calculated by using the calibration curve from the standards with known concentrations. Nitrobenzene-D5 was corrected in all samples except for week 1, 10°C because the calibration curve was non-linear, indicating a bench error. For two runs of samples, the concentration of naphthalene-D8 was absent or extremely high, indicating bench errors. To correct, the naphthalene-D8 correction factors for each jar were averaged over all weeks and the average was used for the two runs with errors. The ion ratios from nitrobenzene-D5 were consistent throughout, indicating that the machine was producing consistent data. Finally, I divided the total mg of DRO in extract by sample wet-weight to get concentration of DRO mg/kg wet-weight. These final values were used to calculate percent loss of DRO.

Genetic verification of fungal identity

To verify whether *Pleurotus ostreatus* persisted to the end of the study, I extracted DNA from fungal tissue or substrate in all of the mycelium-inoculated jars using a FastDNA Spin Kit for Soil. Ian Herriot amplified the fungal ITS sequence with primers ITS1F and ITS4 using PCR, then I ran it on a 1.5% Tris-Borate-EDTA gel. The ITS segment varies in size by fungal species, so the size of the PCR product was used as a preliminary indicator of the presence of *Pleurotus ostreatus*. To verify the presence or absence of *Pleurotus ostreatus,* the PCR product was sequenced using a Sanger sequencer by Ian Herriot.

Statistical analyses

The percent DRO loss from week one to week 16 was calculated. DRO loss was also plotted for each treatment type and fit to a negative exponential equation, and the percent loss was calculated from the equations. Percent loss data were grouped into inoculated vs. uninoculated and straw vs. sawdust for each temperature, with six values in each group. The

Shapiro-Wilk test was used to check for normality. Data were normally distributed, so analysis of variance (ANOVA) tests were performed using R i386 3.1.1 to determine significance of DRO degradation between different treatments and temperatures.

Results

Over the 16 weeks of the evaluation period of this study, average percent loss of total DRO occurred in all treatments. DRO losses were 22-28% at 4°C, 41-55% at 10°C, and 91-92% at 25°C. Average percent loss of DRO across the three replicates was greater in all inoculated mesocosms than uninoculated. However, the differences were not statistically significant at the 95% confidence level (Table 4). In comparing straw treatments to sawdust, straw mesocosms had slightly greater average percent DRO loss at 4°C and 10°C, but less at 25°C. Again, none of these comparisons were significant at the 95% level (Table 5).

Table 4. Average percent loss in inoculated and uninoculated treatments between 1 and 16 weeks.

	Treatments	DRO loss (mg/kg)*	Standard Err (mg/kg)	p value	Percent loss	Standard Error	p value
4°C	Sterile substrate	5.75E+02	1.36E+02	0.1319	22.2%	5.2%	0.455
	Inoculated	6.85E+02	1.52E+02		28.0%	5.4%	
	Just soil	2.68E+01			0.9%		
10°C	Sterile substrate	1.27E+03	1.80E+02	0.213	40.7%	6.1%	0.236
	Inoculated	1.69E+03	2.98E+02		55.1%	9.7%	
	Just soil	1.15E+03			35.9%		
25°C	Sterile substrate	4.34E+03	2.08E+02	0.655	91.5%	1.1%	0.735
	Inoculated	4.53E+03	2.46E+02		92.2%	1.8%	
	Just soil	2.23E+03			80.7%		

[a]The initial concentrations in this study averaged 2640 mg/kg at 4°C, 3147 at 10°C, and 4142 mg/kg at 25°C.

Table 5. Average percent loss in straw vs. sawdust treatments in 16 weeks.

	Treatments	DRO loss (mg/kg)	Standard Err (mg/kg)	p value	Percent loss	Standard Error	p value
4°C	Straw	6.54E+02	1.65E+02	0.5736	25.8%	6.0%	0.946
	Sawdust	6.07E+02	1.24E+02		24.9%	4.8%	
	Just soil	2.68E+01			0.9%		
10°C	Straw	1.60E+03	2.83E+02	0.682	51.3%	9.3%	0.582
	Sawdust	1.36E+03	2.31E+02		44.4%	7.8%	
	Just soil	1.15E+03			35.9%		
25°C	Straw	4.31E+03	1.22E+02	0.461	91.6%	1.8%	0.835
	Sawdust	4.57E+03	2.93E+02		92.1%	1.2%	
	Just soil	2.23E+03			80.7%		

[a]The average percent loss of DRO was positively correlated with temperature. The 25°C treatments averaged 3.66 times less diesel after 16 weeks than the 4°C treatments (Figures 1-3).

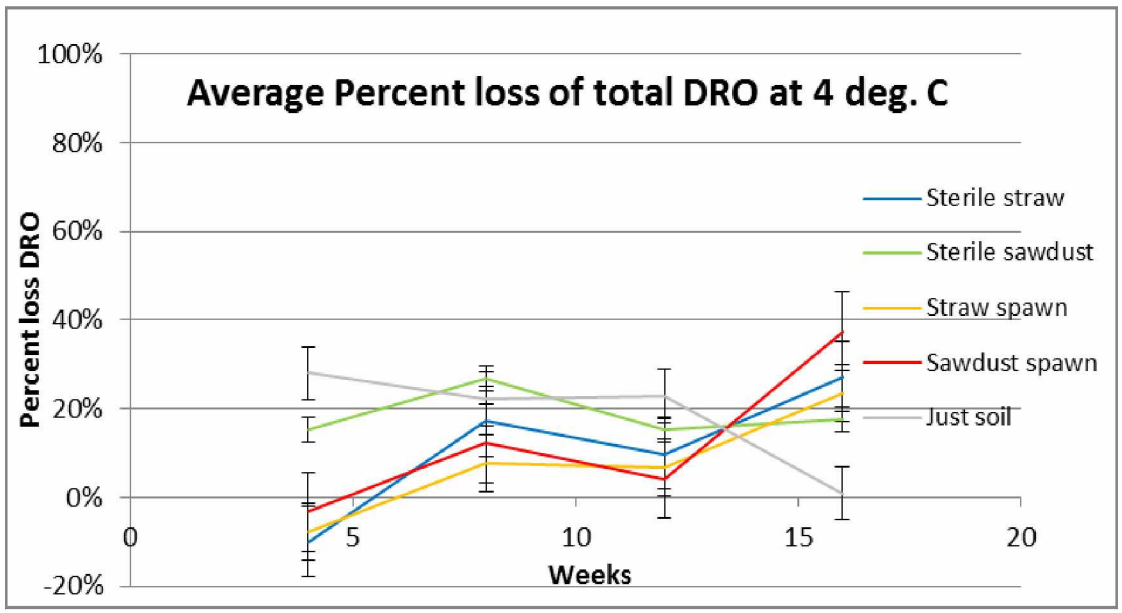

Figure 1. Percent DRO Loss at 4°C.

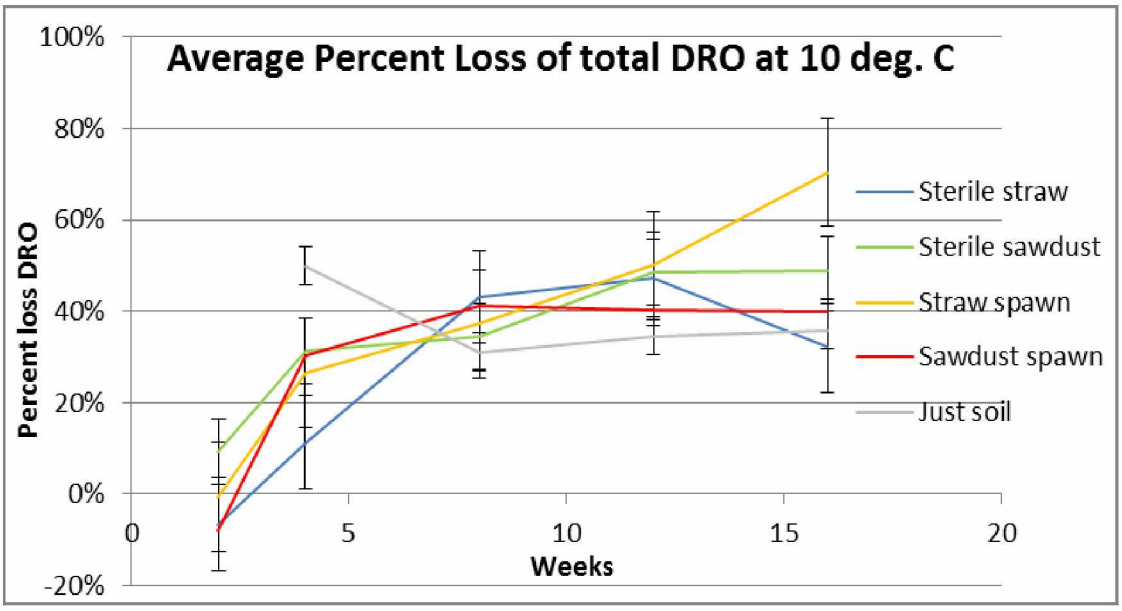

Figure 2. Percent DRO Loss at 10°C.

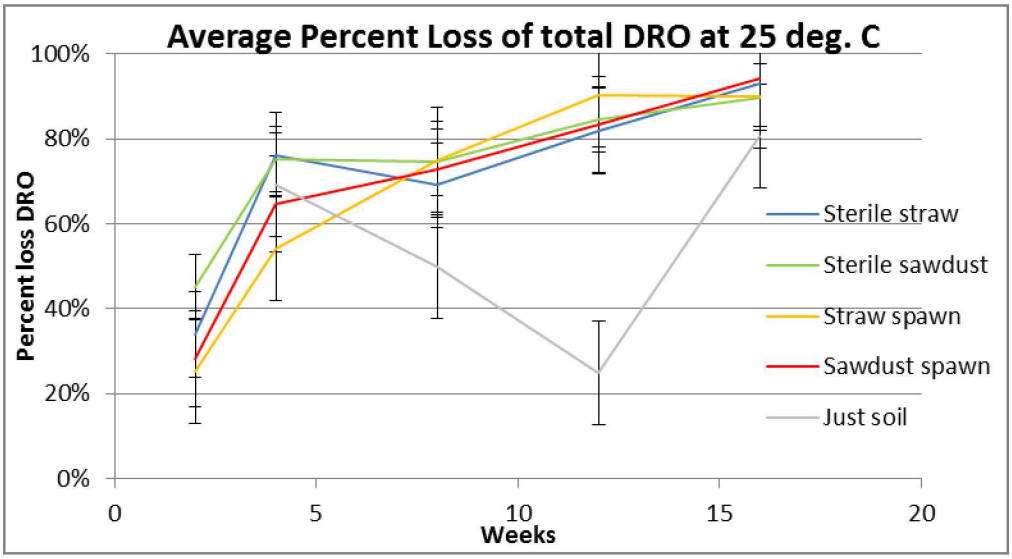

Figure 3. Percent DRO Loss at 25°C.

Variation of DRO at each temperature in unamended soil was least consistent of all treatments. In some cases, measurements at later weeks were higher than previous weeks. I believe this

variation was primarily due to the lack of replicates, and variation of diesel concentration in the mesocosms and resulting sample effect, as discussed in the next section.

Survival and diameter growth of mycelia displayed relatively little inhibition in relation to increasing diesel concentration (Figure 4).

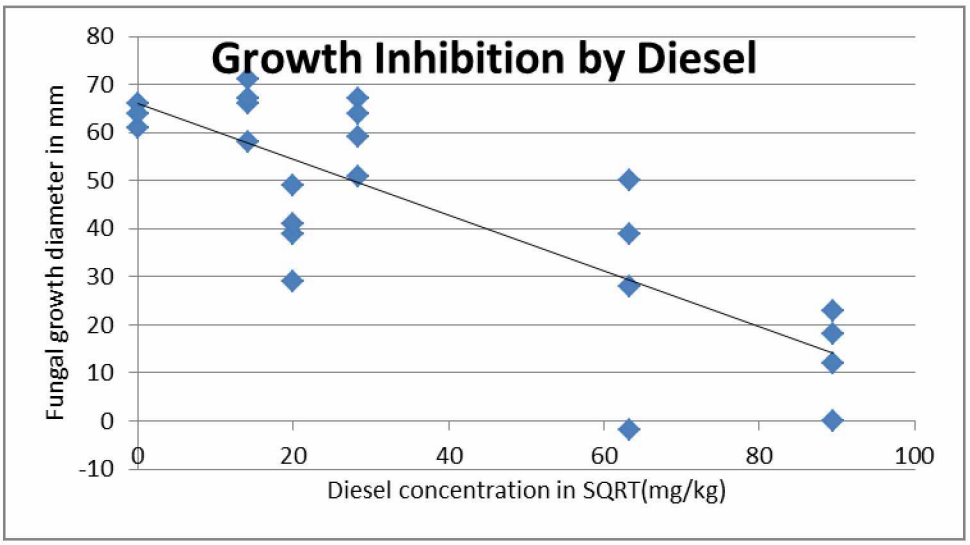

Figure 4. Fungal tolerance to varying concentrations of diesel.

Concentration of diesel added to the growth media ranged from about 200 - 20,000 ppm. The concentration of diesel in the soil from the Kaltag School Oil Seep ranged from 2,890 to 5,740 ppm.

Based on the mycelial growth ranking system, I observed that the mycelium initially grew well at all temperatures (Figure 5).

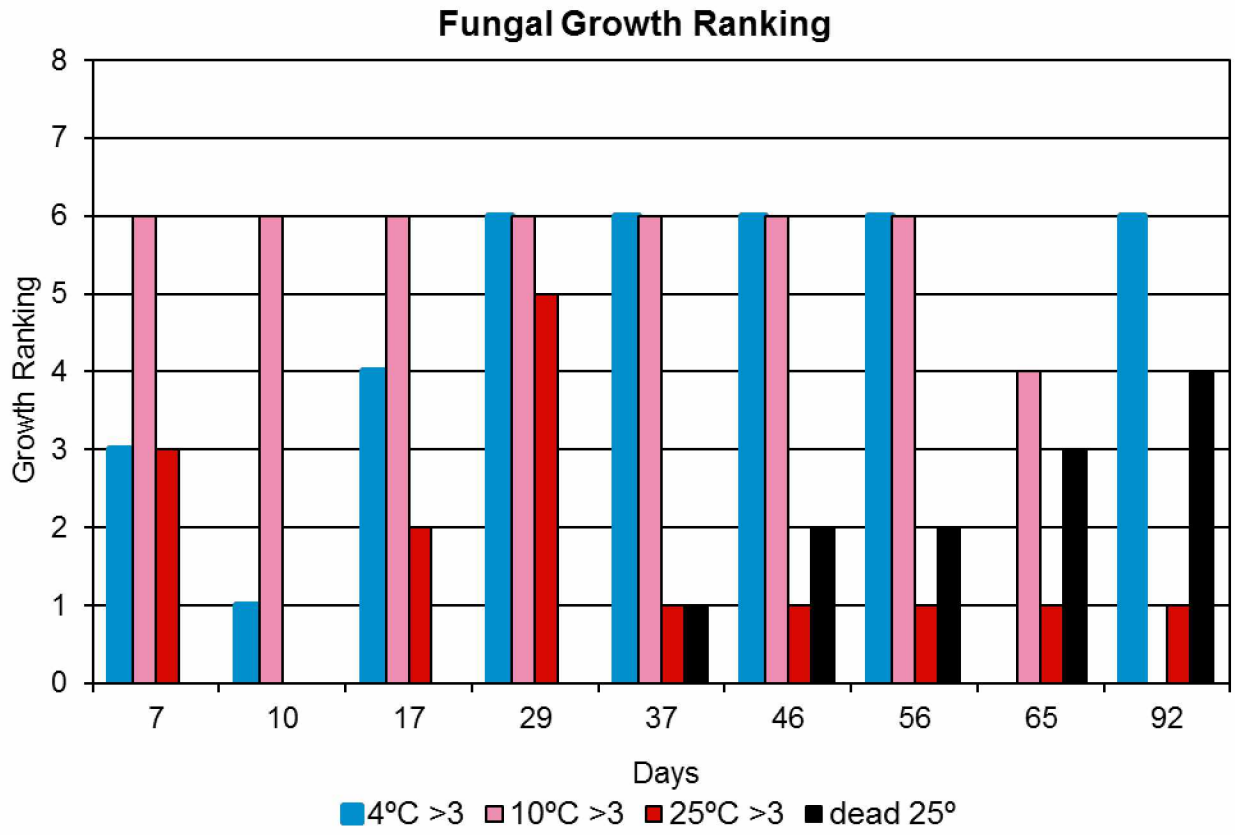

Figure 5. Fungal growth. The number of jars exhibiting a growth rating greater than or equal to 3, and the number of jars in which the fungal mycelium had died.

In the 4ºC treatment, the mycelium became thick and fluffy, but never formed fruiting bodies. At 10ºC, mycelium grew well, and all jars produced "pinheads" at week 8, which matured to fruiting bodies in two of the jars at week 12 (Figure 6).

Figure 6. Straw spawn A 10ºC, week 8.

Mycelium grew rapidly at 25ºC until about week 8. During week 8, the jars at 25ºC began to become invaded by unidentified mold, and much of the *P. columbinus* mycelium appeared to die (Figure 5, Figures 7-9,).

Figure 7. Straw spawn A 25ºC, week 1.

Figure 8. Straw spawn A 25ºC, week 2.

Figure 9. Straw spawn B 25°C, week 9.

In four of the inoculated mesocosms, incubated at 25°C, no *P. columbinus* mycelium was visible at the end of the incubation. The gel electrophoresis product contained no fungal DNA for these same four mesocosms (Figure 10), and the diagnostic sequencing standard used to determine fungal DNA were inconclusive.

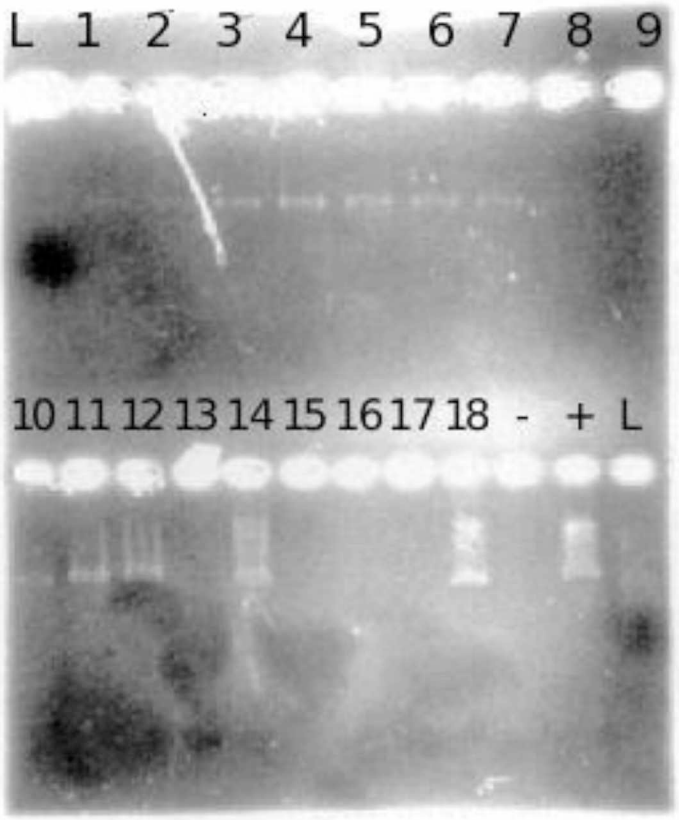

Figure 10. Gel image of DNA extracted from inoculated jars at the end of incubation. Numbers 1-18 correspond to straw spawn A-C at 4°C, sawdust spawn A-C at 4°C, straw spawn A-C at 10°C, sawdust spawn A-C at 10°C, straw spawn A-C at 25°C, and sawdust spawn A-C at 25°C.

By contrast, mesocosms incubated at 4°C and 10°C contained visible *Pleurotus columbinus* in 10 out of 12 cases. Gel electrophoresis confirmed presence of *Pleurotus sp.* DNA in all 10 cases (Figure 10).

Discussion

Although DRO loss occurred in all treatments, the contribution of inoculation and substrate was not as obvious as the strong influence of increasing temperature. The 91%-92% loss at 25ºC, while considerable, did not meet the DEC recommended cleanup level for arctic soils of 200 mg/kg (ADEC, 2000), but could with more time.

Aside from the strong influence of temperature, I searched for subtle effects of substrate and inoculation on DRO loss. A great deal of variability in the results is most likely caused by soil heterogeneity and sampling effect. Because of this great variability, we cannot conclude that inoculation enhanced DRO loss. However, greater percent DRO loss in inoculated than uninoculated mesocosms at 10ºC, though only significant with 76.4% confidence, leaves the possibility of fungal influence open. Greater homogenization of the mesocosm might reduce the variability caused by random sampling of soil for analysis, and allow any subtle level of enhancement that might be caused by fungal inoculation to be determined more readily.

Our results give little or no basis to believe that substrate difference (sawdust versus straw) would preferentially enhance DRO loss. This suggests that there is no basis for preferring a particular material for remediation at rural Alaska remediation sites; whatever is locally abundant may be used.

The large disparity of DRO loss at 25ºC treatment versus 4ºC treatment strongly suggests that treatments that increase soil temperature at spill sites may be the most effective remediation method. A key question remains whether the enhanced loss at higher temperatures occurs primarily due to enhanced microbial activity, including fungi, or primarily from the well-established phenomenon of increased volatilization at higher temperatures (Atlas, 1981). The low percent loss of DRO at 4ºC compared to the higher incubation temperatures in this study is

consistent with lower volatility and bioavailability of diesel reported at cold temperatures (Yang *et al.*, 2009).

The relatively high levels of diesel concentration associated with diesel toxicity suggests that this species could survive across a wide range of contamination levels that could be found in Alaska (Figure 4). Therefore, we cannot rule out the use of *P. columbinus* for DRO remediation based on diesel sensitivity, but concentration of diesel may influence the ability of *P. columbinus* to degrade it. *Polyporus sp.* exhibited greater crude oil degradation at 1000 ppm oil concentrations and lower degradation at 15,000 ppm (Hadibarata and Tachibana, 2009). *P. ostreatus* also exhibited greater crude oil degradation at lower concentrations (Zitte *et al.,* 2012), although this study did not include statistical analysis. However, the highest DRO loss in this mesocosm study occurred at highest concentrations of diesel, not the lowest.

Ten degrees Celsius is the optimal growth temperature both for *P. columbinus* (Personal communication) and for fungi from cold, alpine soils (Mair *et al.,* 2013). If this relationship is applicable in conditions similar to this study, degradation in inoculated treatments would have been optimized at 10°C. However, the physiology of white-rot fungi may play an important role in temperature-dependent degradation of DRO. A profile of ligno-cellulolytic enzymes during growth and fruiting of *P. ostreatus* found that the main enzymes that degrade hydrocarbons, laccases and Mn-peroxidases (Novotný *et al.,* 2004), were highest during vegetative growth and sharply decreased during fruiting (Elisashavili *et al.,* 2008). If this relationship applies to *P. columbinus*, then most of the fungal DRO degradation in the 10°C mesocosms of this study would have occurred before fruiting at week 8. The general flattening of the percent DRO loss curve at week 8 in the sawdust spawn treatment is consistent with such a mechanism. However, the percent DRO loss continued to increase after week 8 in the straw spawn treatment (Figure 2).

Further careful examination could determine whether this divergence in DRO degradation under these conditions might be due to a substrate-specific effect.

It is not clear that increasing temperatures would improve prospects of mycoremediation in the field. In fact, observations from this study suggest that competition with other microbes is likely to displace *P. columbinus* at such temperatures (Figures 8 and 5). With an optimal growth temperature of 10ºC and a sensitivity to warm temperatures, *Pleurotus columbinus* is a psychrophile (Margesin and Schinner, 2001). White-rot fungi can be hampered by competition with native microbiota if introduced directly to the soil, because soil is not their natural habitat (Lladó *et al.,* 2013). However, fungi can also have a synergistic effect with indigenous bacteria, with cooperation or competition leading to greater or less breakdown of toxic compounds (Federici *et al.,* 2007; Li *et al.*, 2008; Tornberg *et al.,* 2003; Atlas, 1995).

Soil nutrient analysis

In most natural environments, soil copper ranges from 2 to 100 ppm (Schulte and Kelling, 1999). However, the soil used in this study contained only 1.3 ppm of copper. As a result, it is possible that copper could have been a limiting factor for laccase synthesis in the Kaltag School Oil Seep soil mesocosms. Low levels of copper (4 mM) inhibited laccase production in *Pleurotus pulmonarius* (Tychanowicz *et al.,* 2006). In addition to laccase, another important enzyme for mycoremediation is Mn-peroxidase, which can be limited by manganese presence in the substrate. The manganese concentration in the Kaltag School Oil Seep soil used in this study was 104 ppm, well within the normal soil range of 40 to 900 ppm (ATSDR, 1997), and thus was unlikely to be limiting for the Mn-peroxidase reaction. Additional test data collected from the *P. columbinus* culture used in this study confirmed that two Mn-peroxidase

genes were present, but the genes for three laccase enzymes were not detected*. Although we cannot conclude that the laccase genes were not present, if they truly are absent, it could decrease the fungus' ability to degrade diesel. *RAHI student Ronin Ruerup performed a qPCR using three laccase and two Mn-peroxidase primers on this *P. columbinus* (data not shown).

Ambient temperature at the treatment site can vary from the temperature at the fungal-substrate interface due to the thermogenesis associated with organic matter decomposition. In studies that used large quantities of contaminated soil and fungal spawn, there was a thermogenic effect from organic matter decomposition (Guerin, 2000; Semple *et al.,* 2001). My quart-size, glass treatment jars did not insulate enough to allow thermogenesis that would occur in a larger reaction. This suggests that soil amendments at cold soil remediation sites in Alaska might be configured to maximize this source of heat to increase DRO degradation. However, the method of application of soil organic amendments could have a bulking effect, preventing soil from reaching thermogenesis (Semple *et al.,* 2001). I recommend any field scale clean-up using microbial degradation in sub-arctic regions to use techniques to maximize heat capture. The "biopile" design or a clear plastic or Reemay ground cover are some cost effective ways to increase soil temperature (Filler and Carlson, 2000; Marion and Pidgeon, 1992).

Key points and recommendations

As research and experience strengthens the evidence for bioremediation, regulatory agencies are more likely to adopt it as a standard technology (Boopathy, 2000). More care should be taken to thoroughly homogenize and sample field scale mycoremediation treatments, especially when measuring total petroleum hydrocarbons instead of specific PAHs. Toxicity tests at the beginning and end of field studies are recommended in order to better assess the success of the treatment.

Increasing the temperature of the mesocosms in this study produced a greater percent loss of DRO than inoculation with *P. columbinus*, suggesting that increasing temperature at remediation sites and should be the first priority for remediation of diesel spills in sub-arctic soils. A larger experimental set-up allowing for the thermogenic effect, like a biopile, could significantly increase diesel loss in northern climates. If fungal inocula were more widely available, as spent oyster mushroom spawn from mushroom farms is in other states, it would be more economical to use as an amendment. From my results, I have not ruled out a subtle effect from *P. columbinus* inoculation. Applying these results to test mycoremediation with the biopile technique logically follows.

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General Conclusion

The relatively new technique of mycoremediation has potential for enhancing the breakdown of toxic environmental organic contaminants. New species of fungi are still being discovered as potential agents for mycoremediation of a growing list of contaminants. The ability of fungal spawn to extend a mycelial network through contaminated substrate may be particularly useful for connecting heterogeneously distributed contaminants. Volatilization due to temperature plays a more important role in diesel loss than fungal inocula, but for non-volatile contaminants, mycoremediation has greater potential. Fungi may be especially valuable in synergy with bacteria, in cases where the contaminant is too recalcitrant (e.g. high molecular weight PAHs) to be easily degraded by bacteria. However, it can be challenging for white rot fungi to establish in soil, and they require a lignocellulosic substrate.

Appendix

Leigh Lab DRO
SOP Updated
9/1/2014

Leigh Lab
Diesel Range Organics and Polycyclic Aromatic Hydrocarbons Extraction and Analysis SOP

Introduction

This document provides a standard operating procedure for determining concentrations of diesel range organics (DRO) in soil samples using gas chromatography/mass spectrometry (GC/MS). The extraction procedures are based on the Alaska Method AK 102 for DRO, but have been streamlined for extracting large numbers of samples in a short amount of time where concentrations and soil types do not vary greatly. The analysis is significantly different than AK 102 in that it utilizes a mass spectrometer (MS) instead of a flame ionization detector (FID). GC/MS was selected based on the available equipment in the research laboratory.
The following sections are intended for internal use to guide analysts through sample extraction, analysis, and data processing and reporting, with technical assistance from chemist Shane Billings of the UAF Water and Environmental Research Center. It is not intended as a stand-alone method, and may need to be modified based on the nature of the soil samples to be analyzed. The user should be proficient in basic analytical chemistry techniques (diluting standards, pipetting, weighing, etc.) and should be familiar with the methods upon which this SOP is based (AK 102, EPA Method 8270D) and basic analytical-method terminology.

Safety

In addition to basic laboratory safe practices, the following safety considerations are highlighted:

- Methylene chloride (CH_2Cl_2) is highly volatile, can dissolve/penetrate nitrile gloves, and is hazardous to your health. Conduct all procedures involving CH_2Cl_2 in the fume hood, wearing polyvinyl alcohol (PVA), butyl, or viton gloves.
- A subset of samples will be oven-dried for determination of percent moisture – as soil samples are contaminated with DRO, ensure that the oven is adequately vented to the outside.

Laboratory Equipment, Reagents, and Supplies

Laboratory Equipment

- GC/MS
- Shaker table that can fit in fume hood
- 100 mL and 250 mL volumetric flasks (note: it is good to have a set of 6x 100 mL flasks

for preparing calibration standards, and several 250 mL flasks for preparing surrogates, internal standards, etc.)
- 1 mL pipette
- 20 µL pipette

Reagents

- Methylene chloride (50 mL per sample, plus additional 50% for blanks, standards, rinses, etc.)
- Methanol (for preparing surrogate)
- Nitrobenzene-d5 (enough to prepare a 1,000 mg/L internal standard)
- Naphthalene-d8 (enough to prepare a 1,000 mg/L surrogate)
- Diesel fuel (or #2 heating oil) for DRO calibration standards
- PAH standard mix (in CH_2Cl_2) for PAH calibration standards
- Sodium sulfate (Na_2SO_4; 10 g per sample) for drying samples

Supplies

- 4 oz. amber glass jars with Teflon-lined lids (~50; can be washed and reused)
- 40 mL VOA vials (one per sample, plus 50% for standards, etc.)
- ~5 mL bulb-type glass pipettes (one per sample)
- 1 mL pipette tips (2 per sample)
- 20 µL pipette tips (1 per sample)

Standard Preparation

DRO Stock Solution: ~ 16 g/L

- Tare a 250 mL volumetric flask on an analytical balance.
- Add 5 mL of diesel fuel – accurately record mass (density is about 0.8 g/mL)
- Dilute to volume with methylene chloride.
- Store in amber glass bottle with Teflon-lined lid

DRO Calibration Standards: 40 mg/L; 160 mg/L; 800 mg/L; 1600 mg/L

- Transfer the following volumes of the stock solution into 100 mL volumetric flasks
  - 0.25 mL
  - 1.0 mL
  - 5.0 mL
  - 10.0 mL
- Add 1.00 mL of ~1,000 mg/L naphthalene-d8 standard to each flask (equivalent of the 0.5 mL added to each soil sample extracted with 50.0 mL of methylene chloride)
- Dilute to volume with methylene chloride
- Store each standard in three 40-mL VOA vials (allowing for backups if cross-contamination were to occur)

Naphthalene-d8 Surrogate: ~1,000 mg/L

- Transfer approximately 250 mg of naphthalene-d8 (solid) into a 250 mL volumetric flask
- Dilute to volume with methanol

Note: concentration can be approximate as the naphthalene-d8 recovery is calculated only in relative terms (relative to the amount of surrogate added to calibration standards)

Nitrobenzene-d5 Internal Standard

- Tre a 100 mL flask on an analytical balance
- Add ~100 mg of nitrobenzene-d5 (oily liquid) to flask
- Dilute to volume with methylene chloride

Note: as with the surrogate, concentration can be approximate as the nitrobenzene-d5 is only used in relative terms (to correct for variability in injection amount)

Section 1. Sample Extraction

Alaska Method AK 102 notes that "any sample extraction technique that meets the quality assurance requirements specified in Section 10 and Table 1 of this method may be used." The extraction method presented in this SOP is sufficient for extracting DRO and PAH from soil samples that contain non-trace quantities of target analyses, inefficiencies in the extraction are accounted for by correcting the result, as based on surrogate recovery. Our test batch indicates this extraction method meets quality assurance requirements (AK 102: surrogate recovery of 50 – 150 %).

- Weigh between 10 g and 12 g of soil into a 4 oz. amber glass jar using a stainless steel spoon. Record sample mass accurately.
- Add 10 g of sodium sulfate (drying agent) to the soil and stir to mix thoroughly. Sample should become loose and grainy. Amount of drying agent may be adjusted according to average moisture content of the samples.
- Pipette 0.5 mL of ~1,000 mg/L naphthalene-d8 surrogate onto soil sample. Stir to thoroughly incorporate surrogate into the soil sample.
- Add 50.0 mL of methylene chloride to the sample using a repipetter in the fume hood.
- Place sample on shaker table. Turn shaker table on and set to a speed just below where solvent reaches the jar lid.
- Allow samples to shake for 3 hours.
- Turn off shaker table and allow samples to settle for 10 minutes.
- Using disposable non-volumetric glass transfer pipettes (with rubber bulb), transfer at least 10 mL of sample extract into a 40-mL VOA vial. Try to avoid transferring any solids.
- Prepare QC samples as described in the following section.
- Store extracts in a freezer at < -10 °C.

Data Quality Objectives

The following data quality objectives (DQOs) are defined for this SOP, and are based on standard methods and ADEC data-quality guidance.

Practical Quantitation Limit

A practical quantitation limit (PQL) must be determined for each analyte (DRO and individual PAHs).

DRO Analysis

This method uses gas chromatography and mass spectrometry to quantitate DRO concentrations in the C_{10} to C_{25} range. Samples are analyzed in total ion current mode. The following specifies the instrument parameters for the analytical method.

Section 2. Extract preparation

Pipette 1.00 mL of clean methylene chloride (blank), each calibration standard, and each sample into a separate GC vial. Add 20 µL of ~1,000 mg/L nitrobenzene-d5 internal standard to each vial; after each addition, take up the sample into the pipette tip and empty several times to ensure all 20 µL get into the sample – discard pipette tip after each sample prep. Load GC vials onto autosampler tray and program a new sequence on the instrument. Include QC samples described in the section above.